FORM PTO-1390 (Modified) (REV 11-98) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

# TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

| ATTORNEY'S DOCKET NUMBER | U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR |
|---|---|
| PF3623USW | 09/936506 |

| INTERNATIONAL APPLICATION NO. | INTERNATIONAL FILING DATE | PRIORITY DATE CLAIMED |
|---|---|---|
| PCT/EP 00/02031 | 9 March 2000 | 11 March 1999 |

TITLE OF INVENTION
**EXPRESSION**

APPLICANT(S) FOR DO/EO/US
**Herve Jean-Clement COSTE; Jonathan Henry ELLIS**

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
   a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☒ has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☒ A copy of the International Search Report (PCT/ISA/210).
8. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☐ have not been made and will not be made.
9. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
10. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
11. ☒ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).

**Items 13 to 20 below concern document(s) or information included:**

13. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☒ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☒ Certificate of Mailing by Express Mail
20. ☒ Other items or information:

PCT Request



PCTUS1/REV03

Express Mail Label No.:
EL395942773US

| U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR) | INTERNATIONAL APPLICATION NO. | ATTORNEY'S DOCKET NUMBER |
|---|---|---|
| 09/936506 | PCT/EP 00/02031 | PF3623USW |

21. The following fees are submitted:.

**BASIC NATIONAL FEE ( 37 CFR 1.492 (a) (1) - (5)) :**

| | | CALCULATIONS PTO USE ONLY |
|---|---|---|
| ☐ | Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2) paid to USPTO and International Search Report not prepared by the EPO or JPO ..... | **$1,000.00** |
| ☒ | International preliminary examination fee (37 CFR 1.482) not paid to USPTO but Internation Search Report prepared by the EPO or JPO ..... | **$860.00** |
| ☐ | International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO ..... | **$710.00** |
| ☐ | International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) ..... | **$690.00** |
| ☐ | International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) ..... | **$100.00** |
| | **ENTER APPROPRIATE BASIC FEE AMOUNT =** | **$860.00** |
| | Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)). | **$0.00** |

| CLAIMS | NUMBER FILED | NUMBER EXTRA | RATE | |
|---|---|---|---|---|
| Total claims | 34 - 20 = | 14 | x $18.00 | **$252.00** |
| Independent claims | 1 - 3 = | 0 | x $80.00 | **$0.00** |
| Multiple Dependent Claims **(check if applicable)**. | | | ☐ | **$0.00** |
| | **TOTAL OF ABOVE CALCULATIONS =** | | | **$1,112.00** |
| | Reduction of 1/2 for filing by small entity, if applicable. Verified Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28) **(check if applicable)**. ☐ | | | **$0.00** |
| | **SUBTOTAL =** | | | **$1,112.00** |
| | Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)). + | | | **$0.00** |
| | **TOTAL NATIONAL FEE =** | | | **$1,112.00** |
| | Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) **(check if applicable).** ☐ | | | **$0.00** |
| | **TOTAL FEES ENCLOSED =** | | | **$1,112.00** |
| | | | | **Amount to be: refunded** $ |
| | | | | **charged** $ |

☐ A check in the amount of to cover the above fees is enclosed.

☒ Please charge my Deposit Account No. **07-1392** in the amount of **$1,112.00** to cover the above fees.
A duplicate copy of this sheet is enclosed.

☒ The Commissioner is hereby authorized to charge any fees which may be required, or credit any overpayment to Deposit Account No. **07-1392** A duplicate copy of this sheet is enclosed.

**NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.**

SEND ALL CORRESPONDENCE TO:

**GlaxoSmithKline**
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**Five Moore Drive, P.O. Box 13398**
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SIGNATURE

**Elizabeth Selby**
NAME

**38,298**
REGISTRATION NUMBER

**September 11 , 2001**
DATE

PF3623USW

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

| | |
|---|---|
| In re Application of: | COSTE, et al |
| International Application No.: | PCT/EP00/02031 |
| International Filing Date: | March 9, 2000 |
| **Title:** | **EXPRESSION** |

Commissioner for Patents
Washington, D.C. 20231

Attention: Box PCT/DO/EO/US

**FIRST PRELIMINARY AMENDMENT**

Sir:

The above-identified application is being transmitted herewith for entry into the U.S. National Phase under Chapter II of the PCT. For the purposes of adding the priority information, please amend the application as follows:

**In the Abstract:**

Please substitute the attached Abstract, which has been placed on a separate piece of paper according to US practice.

**In the Specification:**

On the first line of the specification, after the Title, please add:

--This application is filed pursuant to 35 U.S.C. § 371 as a United States National Phase Application of International Application No. PCT/EP00/02031 filed March 9, 2000, which claims priority from GB9905498.3 filed March 11, 1999 --

**In the Claims:**

Please amend the claims as follows:

3. A DNA molecule according to claim 1 wherein said untranslated region has a ΔG of below –10kCal/mol.

4. A DNA molecule according to claim 1 wherein said sequence has a ΔG that is below –30kCal/mol.

5. A DNA molecule according to claim 1 wherein said sequence has a ΔG that is below –40kCal/mol.

6. A DNA molecule according to claim 1 wherein said untranslated region has a ΔG of below –50kCal/mol.

7. A DNA molecule according to claim 1 wherein expression of said polypeptide is heat shock responsive.

8. An RNA molecule obtainable by transcribing a DNA molecule according to claim 1.

9. A vector comprising a DNA molecule according to claim 1.

10. An expression system according to claim 1, comprising a DNA molecule or a vector comprising said DNA molecule.

16. A method of obtaining a polypeptide comprising expressing the polypeptide using an expression system according to claim 10 and, optionally, purifying the polypeptide.

18. A method of treating a deficiency in the expression of a polypeptide, comprising providing a patient with a DNA molecule as claimed in claim 1 which encodes

said polypeptide, a vector comprising said DNA molecule, or a cell comprising said DNA molecule or vector.

19. A method of treating a deficiency in the expression of a polypeptide, comprising providing a patient with a DNA molecule as claimed in claim 1 wherein said molecule is provided in a manner to allow it to become operably linked with a sequence already present in the patient which encodes said polypeptide.

20. A method of treating a disorder (e.g. an infection) treatable by providing an increased immune response, comprising providing a patient with a vaccine comprising a DNA molecule as claimed in claim 1 or a vector comprising said DNA molecule.

21. A method according to claim 18, wherein a DNA molecule or vector is provided under conditions allowing it to integrate within the patient's genome.

25. A pharmaceutically acceptable composition comprising a DNA molecule according to claim 1, an RNA molecule obtainable by transcribing said DNA molecule, or an expression system comprising said DNA molecule.

26. A vaccine comprising a DNA molecule according to claim 1, or a vector comprising said DNA molecule.

27. The use of a DNA molecule according to claim 1, of an RNA molecule obtainable by transcribing said DNA molecule, of a vector comprising said DNA molecule, or of an expression system comprising said DNA molecule, in achieving increased expression of a polypeptide.

28. A DNA molecule according to claim 1 for use in therapy.

30. A DNA molecule according to claim 28 when administered by particle bombardment.

31. A DNA molecule according to claim 28 for use in achieving an increased immune response.

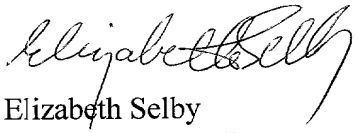
32. A method of therapeutic or prophylactic vaccination comprising administering an effective amount of a DNA molecule as claimed in claim 1.

34. A method according to claim 32 for use in achieving an increased immune response.

**REMARKS**

Currently claims 1-34 are pending. Claims 3-10, 16, 18-21, 25-28, 30-32 and 34 have been amended to place them in form appropriate to US practice and to reduce the filing fee by removing multiple dependency. Applicants have attached an abstract on a separate sheet of paper as required by US practice. Applicants have amended the specification for purposes of adding the priority information.

Respectfully submitted,



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Marked-Up Copy of Pending Claims

3. (Amended) A DNA molecule according to claim 1 **[or 2]**wherein said untranslated region has a ΔG of below –10kCal/mol.

4. (Amended) A DNA molecule according to claim 1 **[any preceding claim]** wherein said sequence has a ΔG that is below –30kCal/mol.

5. (Amended) A DNA molecule according to claim 1 **[any preceding claim]** wherein said sequence has a ΔG that is below –40kCal/mol.

6. (Amended) A DNA molecule according to claim 1 **[any preceding claim]** wherein said untranslated region has a ΔG of below –50kCal/mol.

7. (Amended) A DNA molecule according to claim 1 **[any preceding claim]** wherein expression of said polypeptide is heat shock responsive.

8. (Amended) An RNA molecule obtainable by transcribing a DNA molecule according to claim 1**[any of claims 1 to 7]**.

9. (Amended) A vector comprising a DNA molecule according to claim 1**[any of claims 1 to 7]**.

10. (Amended) An expression system comprising a DNA molecule according to claim 1 **[any of claims 1 to 7]**, or a vector comprising said DNA molecule **[according to claim 9]**.

16. (Amended) A method of obtaining a polypeptide comprising expressing the polypeptide using an expression system according to claim 10 **[any of claims 10 to 15]** and, optionally, purifying the polypeptide.

18. (Amended) A method of treating a deficiency in the expression of a polypeptide, comprising providing a patient with a DNA molecule as claimed in claim 1 **[any of claims 1 to 7]** which encodes said polypeptide, a vector **[as claimed in claim 9]** comprising said DNA molecule, or a cell comprising said DNA molecule or vector.

19. (Amended) A method of treating a deficiency in the expression of a polypeptide, comprising providing a patient with a DNA molecule as claimed in claim 1 **[any one of claims 1 to 7]** wherein said molecule is provided in a manner to allow it to become operably linked with a sequence already present in the patient which encodes said polypeptide.

20. (Amended) A method of treating a disorder (e.g. an infection) treatable by providing an increased immune response, comprising providing a patient with a vaccine comprising a DNA molecule as claimed in claim 1 **[any of claims 1 to 7]** or a vector comprising said DNA molecule **[as claimed in claim 9]**.

21. (Amended) A method according to claim 18 **[or 19]**, wherein a DNA molecule or vector is provided under conditions allowing it to integrate within the patient's genome.

25. (Amended) A pharmaceutically acceptable composition comprising a DNA molecule according to claim 1 **[any of claims 1 to 7]**, an RNA molecule obtainable by transcribing said DNA molecule **[according to claim 8]**, or an expression system comprising said DNA molecule **[a cell as described in claim 11]**.

26. (Amended) A vaccine comprising a DNA molecule according to claim 1 **[any of claims 1 to 5]**, or a vector comprising said DNA molecule **[according to claim 9]**.

27. (Amended) The use of a DNA molecule according to claim 1 **[any of claims 1 to 7]**, of an RNA molecule obtainable by transcribing said DNA molecule **[according to claim 8]**, of a vector comprising said DNA molecule **[according to claim 9]**, or of an expression system comprising said DNA molecule **[according to claim 10]**, in achieving increased expression of a polypeptide.

28. (Amended) A DNA molecule according to claim 1 **[any one of claims 1 to 7]** for use in therapy.

30. (Amended) A DNA molecule according to claim 28 **[or 29]** when administered by particle bombardment.

31. (Amended) A DNA molecule according to claim 28**[, 29 or 30]** for use in achieving an increased immune response.

32. (Amended) A method of therapeutic or prophylactic vaccination comprising administering an effective amount of a DNA molecule as claimed in claim 1**[any one of claims 1 to 7]**.

34. (Amended) A method according to claim 32 **[or 33]** for use in achieving an increased immune response.

# Expression

The present invention relates *inter alia* to the provision of increased polypeptide expression.

The human Hsp70A gene has been sequenced by Hunt C. and Morimoto R.I.(1985) *Proc.Natl.Acad.Sci. USA* **82**, 6455-6459. ) This gene encodes an mRNA containing a 5'-untranslated region (5'UTR) of 215 bases. As for most of the vertebrate Hsp mRNAs, the base composition of the human Hsp70 5'UTR is rich in guanosine and cytosine (~62%) (Joshi C.P. and Nguyen H.T. (1995) *Nucleic Acids Res.* **23**, 541-549) suggesting that the human Hsp70 5'UTR has a high potential to form secondary stuctures in this region. It is believed that the function of the human Hsp70 5'UTR has never been previously studied.

In contrast, the Drosophila Hsp70 5'UTR has been extensively studied (Di Nocera P.P. and Dawid I. (1983) *Proc.Natl.Acad.Sci.USA* **80**, 7095-7098; Bonner J.J. *et al.* (1984) *Cell* **37**, 979-991; McGarry T.J. and Lindquist S. (1985) *Cell* **42**, 903-911; Hultmark D. *et al.* (1986) *Cell* **44**, 429-438; Lindquist S. and Petersen R. (1990) *Enzyme* **44**, 147-166). The sequence of the Drosophila Hsp70 5'UTR (Ingolia T.D. *et al* (1980) *Cell* **21**, 669-679) has no significant homology with the human Hsp70 5'UTR. The lack of secondary structure in the Drosophila 5'UTR region due to a rich adenosine composition (~50%) allows efficient translation of this mRNA during heat shock (Hess M.A and Duncan R.F. (1996) *Nucleic Acids Res.***12**, 2441-2449).

Experimental studies with both *in vivo* and *in vitro* systems clearly demonstrate that mRNA with a high potential to form stable secondary structures in the 5'UTR tends to be inefficiently translated (Kozac M.(1991) *J. Biol. Chem.* **266**, 19867-19870; Kozac M. (1991) *J. Cell Biol.* **115**, 887-903). Moreover, structural motifs in the 5'UTR can provide sites for the binding of proteins which can act as negative

regulators of translation (Gray N.K. and Hentze M.W. (1994) *EMBO J.* **13**, 3882-3891; Stripecke R. *et al* (1994) *Mol.Cell.Biol.***14**, 5898-5909).

WO94/11521 is directed to providing inducible expression by using a bovine hsp70 promoter. The promoter may be associated with a human or bovine hsp70 5' untranslated region.

Surprisingly the present inventors have now identified a molecule with a high potential to form secondary structures that can provide increased efficiency of translation.

According to the present invention there is provided a DNA molecule that can be transcribed to provide an RNA molecule having an untranslated region that can provide an increased efficiency of translation of a polypeptide (relative to that obtainable when said untranslated region is absent) when operably linked to a coding region encoding said polypeptide; wherein said DNA molecule does not encode a mammalian hsp70.

Preferably the increased efficiency of translation is an increase of at least 10%. More preferably it is an increase of at least 100%. Most preferably it is an increase of at least 500%.

The use of the present invention to provide significantly increased efficiency of translation (and thereby to provide increased expression) contrasts with the invention disclosed in WO94/11521, for example. This discloses the possibility of using a human hsp70 5' untranslated region, but not for obtaining an increased efficiency of translation. In any event, as indicated above, WO94/11521 is specifically directed to the bovine hsp70 promoter and its use in promoting inducible expression. It is preferred that the bovine promoter hsp70 described in WO94/11521 is not used in the present invention. The human hsp70 promoter may for example

be used in the present invention. Promoters which are not hsp promoters may also be used and are often preferred. Further, and in contrast to WO94/11521, heat shock is not required to increase the protein expression in the preswnt invention.

The untranslated region of the present invention is preferably located upstream of the coding sequence of the RNA molecule – i.e. it is a 5' untranslated region (a 5'UTR).

Preferred DNA molecules of the present invention comprise:

a) the sequence:

5'-ataacggctagcctgaggagctgctgcgacagtccactacctttttcgagagtgactcccgttgtcccaa ggcttcccagagcgaacctgtgcggctgcaggcaccggcgcgtcgagtttccggcgtccggaaggaccgagctctt ctcgcggatccagtgttccgtttccagcccccaatctcagagccgagccgacagagagcagggaaccgc-3',

[On transcription this sequence will produce an mRNA molecule having the following 5'UTR :

5'-

auaacggcuagccugaggagcugcugcgacaguccactaccuuuuucgagagugacucccguugucccaa ggcuucccagagcgaaccugugcggcugcaggcaccggcgcgucgaguuuccggcguccggaaggaccg agcucuucucgcggauccaguguuccguuuccagcccccaaucucagagccgagccgacagagagcagg gaaccgc-3']

b) the complement of the sequence given in a); or

c) a sequence having substantial sequence identity with a sequence as defined in a) or b) above.

Thus a DNA molecule having a specific sequence that can be transcribed to provide the untranslated region of the present invention is within the scope of the present invention (see a) above).

The complement of this sequence is also within the scope of the present invention (see b) above) since the DNA molecule will normally be double-stranded. In any event the complement is useful in designing probes or primers or in providing antisense molecules (which can be used to reduce expression if expression levels become too high). Furthermore, cDNA (which is also within the scope of the present invention) will comprise the complement.

DNA molecules having substantial sequence identity with molecules described in a) and b) above may be used in a similar manner to said molecules and are therefore also within the scope of the present invention (see c) above).

The present invention further provides a DNA molecule as defined herein for use in therapy, particularly for use in therapeutic or prophylactic vaccination, preferably when administered by particle bombardment and most preferably for use in achieving an increased immune response. An increased immune response would be an immune response which is greater than that achieved with an equivalent construct which incorporates a promoter of the art, for example CMV immediate early promoter or SV40 promoter.

The UTR of the present invention is preferably capable of providing heat-shock responsiveness to the expression of a coding sequence in a given expression system. However this is not essential since the untranslated region can provide increased expression even in the absence of a heat shock response.

Desirably the untranslated region of the present invention has a G+C content of greater than 50%. More desirably this is greater than 55% or greater than 60%. High G+C contents are often associated with an increased tendency to form stable secondary structures.

Preferred DNA molecules of the present invention are those that can be transcribed to provide an RNA molecule having an untranslated region that can provide an increased efficiency of translation of a polypeptide (relative to that obtainable when said untranslated region is absent) when operably linked to a coding region encoding said polypeptide; wherein said DNA molecule does not encode human hsp70 and wherein said untranslated region has a ΔG of below –10 kCal/mol.

For the purposes of the present invention ΔG can be calculated using the RNA structural prediction program MFOLD ( Zuker M. and Jacobson A.B. (1995) Nucleic Ac. Res. (23) 2791-2798). Predicted ΔG values may be calculated using the program located at the internet: http://mfold1.wustl.edu/ ~ mfold/mRNA/form1.cgi

Preferably ΔG is below –30 kCal/mol or below –40 kCal/mol. More preferably ΔG is below –50 kCal/mol. Generally speaking, the lower the ΔG value, the greater the degree of secondary structure likely for a given polynucleotide region.

Increased translation efficiency can be achieved with the present invention in a wide variety of different systems. Indeed the present inventors have provided a 5'UTR upstream of the coding sequence of two very different reporters (firefly luciferase and chloramphenicol-acetyltransferase) and have demonstrated significantly increased expression of the reporter (5- to 10-fold) in normal transfected cell culture conditions.

This effect has been obtained in two different promoter contexts (HSP- and SV40-promoter) and in various human cell lines (HepG2,Hep3B,HEK293,WI-38). The 5'UTR did not not modify the level of mRNA but increased the efficiency of translation. This pure translational effect and the fact that the heat shock response is a highly conserved mechanism provide evidence in support of the broad applicability of the present invention.

In principle the expression of any given polypeptide can be increased using the present invention. However it is preferred to use the present inventions to increase the expression of polypeptides that are not heat shock proteins. Most preferably the present invention is used in providing increased expression of polypeptides of relatively high commercial or scientific value. It can for example be used to increase the expression of therapeutic polypeptides. These include interferons, hormones (e.g. insulin), interleukins, erythropoietin, tpa, growth factors, etc. The present invention can of course also be used to increase the expression of other polypeptides – e.g. polypeptides useful in the agro-alimentary or cosmetic industries

A further aspect of the present invention is the provision of new vectors. These may be derived by modifying known vectors to include a DNA sequence which, on transcription, provides an untranslated sequence of the present invention. This can be done by recombinant DNA technology or by mutagenesis techniques. Alternatively vectors may be constructed *de novo*.

Vectors can be used for many purposes – e.g. for amplifying, maintaining or manipulating sequences of interest, for the production of desired gene products, for medicinal purposes etc. Vectors (and nucleic acids) of the present invention may be purified and provided in isolated form if desired. They may be provided in a form substantially free of contaminating proteins.

Many different types of vector can be provided, including plasmids, phasmids, cosmids, YACs, PACs and viruses. Viral vectors include bacteriophage vectors. These can be used to generate high titre combinatorial libraries. Using 'phage display many different polypeptides can be expressed (e.g. antibodies/parts thereof). These techniques are described for example by M J Geisow in *Tibtech* **10**, 75-76 (1992) and by D. Chiswell *et al* in *Tibtech* **10**, 8-84 (1992). Other vectors can be used in addition to those described above.

Whatever vectors are used, it is preferred that they include one or more selectable markers - e.g. drug resistance markers and/or markers enabling growth on a particular medium. In some cases a vector will include a marker that is inactivated when a nucleic acid molecule according to the present invention is inserted into the vector. Here there is desirably at least one further marker, which is different from the marker that is inactivated.

Preferred vectors of the present invention may be introduced into a cell that can then be used to express a desired polypeptide (although cell-free expression systems can also be used). For example, polypeptides can be produced by micro-organisms such as bacteria or yeast, by cultured insect cells (which may be baculovirus-infected), by mammalian cells (such as CHO cells) or by transgenic animals that, for instance, secrete the proteins in milk (see e.g. international patent application WO88/00239). Where glycosylation is desired, eukaryotic expression systems are preferred.

Particularly suitable expression systems are cell lines that can divide in culture and that can be maintained in culture over a long period. These are often referred to as immortal cell lines. Preferred cell lines are mammalian or human cell lines.

Various transcriptional and translational control sequences may be used in expression systems of the present invention. These can be operably linked to a coding sequence encoding a polypeptide to be expressed. The control sequences may be heterologous to the coding sequence. Promoter, operator and/or enhancer sequences may, for example, be provided, as may polyadenylation sites, splice sites, stop and start codons, etc. Polypeptides may initially be expressed to include signal sequences. Different signal sequences may be provided for different expression systems. Alternatively, signal sequences may be absent.

Techniques for manipulating nucleic acids, for expressing and purifying polypeptides, etc. are well known to a person skilled in the art of biotechnology. Such techniques are

disclosed in standard text-books, such as in Sambrook *et al* [*Molecular Cloning* 2nd Edition, Cold Spring Harbor Laboratory Press (1989)]; in Old & Primrose [*Principles of Gene Manipulation* 5th Edition, Blackwell Scientific Publications (1994)]; and in Stryer [*Biochemistry* 4th Edition, W H Freeman and Company (1995)].

The present invention is useful in medicine (both in human treatment and in veterinary treatment). It can be used to treat an existing condition or can be used for prophylactic treatment. In particular, the present invention is useful for treating a disorder involving a deficiency in the expression of a polypeptide. It will therefore be appreciated that the present invention can be used in gene therapy, especially for treating disorders arising due to mutations affecting the expression of a single polypeptide (although it is generally applicable and can also be used to treat disorders affecting the expression of a plurality of polypeptides). Gene therapy may be used, for example, in the treatment of cancer, cardiovascular disorders, cystic fibrosis, etc.

Treatment of a disorder involving a deficiency in the expression of a polypeptide can be performed by providing a patient with a DNA molecule of the present invention that encodes said polypeptide, with a vector comprising said DNA molecule, or with a cell comprising said DNA molecule or vector. Expression of the polypeptide within the patient can then be used to compensate for, or at least to reduce the deficiency. The DNA molecule or the vector can be allowed to integrate into a patient's genome.

Suitable techniques for introducing a nucleic acid molecule or vector into a patient include topical application of the 'naked' nucleic acid in an appropriate vehicle. The nucleic acid may be present together with a pharmaceutically acceptable excipient, such as phosphate buffered saline (PBS). One technique involves particle bombardment (which is also known as 'gene gun' technology and is described in US Patent No. 5371015). Here inert particles (such as gold beads coated with a nucleic acid) are accelerated at speeds sufficient to enable them to penetrate a

surface of a recipient (e.g. skin) by means of discharge under high pressure from a projecting device. (Particles coated with a nucleic acid molecule of the present invention are within the scope of the present invention, as are devices loaded with such particles.) Other methods of administering the nucleic acid directly to a recipient include ultrasound, electrical stimulation, electroporation and microseeding. Particularly preferred is the microseeding mode of delivery. This is described in US-5,697,901.

Nucleic acid molecules of the present invention may also be administered by means of specialised delivery vectors useful in gene therapy. Gene therapy approaches are discussed for example by Verme *et al*, Nature 1997, **389**:239-242. Both viral and non-viral systems can be used. Viral based systems include retroviral, lentiviral, adenoviral, adeno-associated viral, herpes viral and vaccinia-viral based systems. Non-viral based systems include direct administration of nucleic acids and liposome-based systems.

A nucleic acid sequence of the present invention may even be administered by means of transformed cells. Such cells include cells harvested from a subject. The nucleic acid molecules of the present invention can be introduced into such cells *in vitro* and the transformed cells can later be returned to the subject. The nucleic acid molecules need not be introduced into the cells as vectors, since non-vector nucleic acid molecules can be introduced. Some such molecules may integrate into nucleic acid already present in a cell by homologous recombination events. A transformed cell may, if desired, be grown up *in vitro* and one or more of the resultant cells may be used in the present invention. Cells can be provided at an appropriate site in a patient by known surgical or microsurgical techniques (e.g. grafting, micro-injection, etc.)

Another way of treating a deficiency in the expression of a polypeptide comprises providing a patient with a DNA molecule that can be transcribed to provide the

untranslated region of the present invention. This molecule can be provided in a manner to allow it to become operably linked with a sequence already present in the patient that encodes said polypeptide.

A further way of treating a deficiency in the expression of a polypeptide comprises providing a patient with an RNA molecule coding for said polypeptide, which RNA molecule is pruducable by transcribing a DNA molecule of the present invention. The RNA molecule can then be translated *in vivo* to provide the polypeptide.

A still further way of treating a deficiency in the expression of a polypeptide, comprises providing a patient with the polypeptide, wherein the polypeptide has been produced using an expression system of the present invention.

The present invention is also useful in providing DNA vaccines. The direct injection of gene expression cassettes into a living host transforms a number of cells into factories for the production of the introduced gene products. Expression of these delivered genes has important immunological consequences and may result in the specific immune activation of the host against expressed antigens. Although vaccines produced by recombinant DNA technology are safer than traditional vaccines, which are based on attenuated or inactivated bacteria or viruses, they are often poorly immunogenic. Placing an untranslated region of the present invention upstream of the coding sequence of a gene to be delivered in a DNA vaccine can significantly increase expression and can therefore increase immunogenicity. Due to the highly conserved mechanism of heat shock response an increase in polypeptide expression can be expected in every tissue where the gene is delivered. DNA vaccines can be designed to prevent viral, bacterial and parasitic infections (e.g. diphtheria, malaria, leishmaniasis, toxoplasmosis, schistosomiasis, cryptosporidiosis, tuberculosis, HIV, HSV, influenza virus, hepatitis A, B and C), but can also be used for treating cancer, immune-related diseases or for contraceptive purposes. All of these applications are within the scope of the present invention.

When used in medicine, the nucleic acid molecules, vectors, polypeptides and cells discussed above will usually be in the form of a pharmaceutically acceptable composition. One or more pharmaceutically acceptable carriers may be present in such a composition. A pharmaceutical composition within the scope of the present invention may be adapted for administration by any appropriate route, for example by the oral (including buccal or sublingual), rectal, nasal, topical (including buccal, sublingual or transdermal), vaginal or parenteral (including subcutaneous, intramuscular, intravenous or intradermal) routes. Different drug delivery systems can be used to administer pharmaceutical compositions, depending upon the desired route of administration. Drug delivery systems are described, for example, by Langer (*Science* **249**, 1527–1533 (1991)) and by Illum and Davis (*Current Opinions in Biotechnology* **2**, 254–259 (1991)). In summary, it will be appreciated that the present invention can be used to manufacture medicaments for use in the treatment of one or more of the disorders discussed herein.

In addition to the uses discussed above, the invention is of broad applicability for research purposes.

Cell transfection is a technique classically used in research to study the function of a polypeptide. Moreover, many cellular screenings are performed on cells transfected so as to express a given polypeptide. This technique is also used to study the function of a promoter using reporters (e.g. luciferase, chloramphenicol-acetyl-transferase, β-galactosidase etc.) Providing an untranslated region of the present invention upstream of the coding sequence of a reporter gene can significantly increase the expression of a polypeptide of interest. The sensitivity of such experiments can therefore be increased.

Increased polypeptide expression is also useful in many other research applications where large amounts of a given polypeptide need to be synthetized. For example it is useful in structural studies (crystallography, NMR, etc), for the production of

antibodies or fragments thereof (which can be used for example in purification or in binding studies), or for high throughput screening.

The present invention is also useful for diagnostic purposes. For example, it can be used to increase the provision of antibodies or fragments thereof useful in diagnosing the presence of a moiety associated with a particular disorder.

The present invention will now be described by way of example only with reference to the accompanying drawings, wherein:

Figure 1 shows the effect of the human Hsp70 5'UTR on the expression of a luciferase reporter driven by the human HSP70 promoter. The human HSP70 promoter was cloned upstream of the coding sequence of the firefly luciferase gene in the absence or presence of the 5' UTR (plasmids A and B respectively). For both plasmids the 3'UTR was the HSP70 3'UTR. HepG2 cells were transfected with these two chimeric constructs and the levels of luciferase were compared either under normal conditions or after a 30 min heat-shock at 42°C.

Figure 2 shows the effect of the human Hsp70 5'UTR on the expression of luciferase in various cell lines. The same constructs (A and B) were transfected in three other human cell lines (Hep3B, HEK293, WI-38) and the luciferase levels were compared under normal cell culture conditions.

Figure 3 shows the effect of the human Hsp70 5'UTR on the translational efficiency of the chloramphenicol-acetyl-transferase mRNA. The SV40 promoter was cloned upstream of the coding sequence of the chloramphenicol-acetyl-transferase (CAT) gene in the absence or presence of the human Hsp70 5' UTR (plasmids H and I respectively). HepG2 cells were transfected with these two constructs and the levels of CAT mRNA and activity were measured.

Figure 4 shows the effect of the human Hsp70 5'UTR on the expression of luciferase in the presence of the SV40 3'UTR. The Hsp70 promoter was cloned upstream of the coding sequence of the luciferase in the absence or presence of the human Hsp70 5'UTR (plasmids C and D respectively), for both plasmids the 3'UTR was the SV40 3'UTR. The constructs (C and D) were transfected in three human cell lines (HepG2, HEK293, Hep3B) and the luciferase levels were compared under normal cell culture conditions.

Figure 5 shows a comparison of the effect of the human Hsp70 5'UTR with the human Grp78 5'UTR on the expression of the luciferase. The SV40 promoter was cloned upstream of the coding sequence of the luciferase in the presence of the human Hsp70 5'UTR (plasmid F) or of the human Grp78 5'UTR (plasmid G) or in the absence of any 5'UTR (plasmid E) . HepG2 cells were transfected with these three constructs and the the luciferase levels were compared under normal cell culture conditions.

Figure 6 shows a comparison of the effect of the FMDV IRES with the human Hsp70 5'UTR on the expression of luciferase in a dicistronic context. The CMV promoter and a first ORF were cloned upstream of the coding sequence of the luciferase in the presence of the human Hsp70 5'UTR (plasmid J) or of the FMDV IRES (plasmid K) . HepG2 cells were transfected with these three constructs and the the luciferase levels were compared under normal cell culture conditions.

Figure 7 is a schematic map of plasmids A to L referred to in the specification.

The following sequences are provided for reference purposes and show the hsp70 5'UTR sequences from various species :

Human HSP70A

Hunt C. and Morimoto R.I. (1985) *Proc.Natl.Acad.Sci. USA* **82**, 6455-6459

ataacggctagcctgaggagctgctgcgacagtccactacctttttcgagagtgactcccgttgtcccaaggcttccca
gagcgaacctgtgcggctgcaggcaccggcgcgtcgagtttccggcgtccggaaggaccgagctcttctcgcggat
ccagtgttccgtttccagcccccaatctcagagccgagccgacagagagcagggaaccgc

Human HSP70B
Schiller et al (1988) *J.Miol.Biol.***203**,97-105

agcagatccggccgggctggcggcagagaaaccgcagggagagcctcactgctgagcgcccctcgacgcgggc
ggcagcagcctccgtggcctccagcatccgacaagaagcttcagcc

Rat HSP70
Mestril,R., Chi,S.H., Sayen,M.R. and Dillmann,W.H.
Biochem. J. 298 Pt 3, 561-569 (1994)

ctcctcctaatctgacagaaccagtttctggttccactcgcagagaagcagagaagcagagcaagcggcgcgttcc
gaacctcgggcaagaccagcctctcccagagcatccccacgcgaagcgcacccttctccagagcataccccagc
ggagcgcacccttccccagagcatccccgccgccaagcgcaaccttccagaagcagaccgcagcgac

Chicken HSP70
Morimoto,R.I., Hunt,C., Huang,S.-Y., Berg,K.L. and Banerji,S.S.
J. Biol. Chem. 261, 12692-12699 (1986)

cggcagatcgcgccgcagacagcagcgagaagcgggcggaggagacgtgactgcgagcgagcaagtgactg
gcggagcgagtggctgactgaccaagaggaatctatcatc

Mouse HSP70
Hunt,C. and Calderwood,S.B.
Gene 87, 199-204 (1990)

aagctactcagaatcaaatctggttccatccagagacaagcgaagacaagagaagcagagcgagcggcgcgttc
ccgatcctcggccaggaccagccttccccagagcatccacgccgcggagcgcaaccttcccaggagcatccctgc
cgcggagcgcaactttccccggagcatccacgccgcggagcgcagccttccagaagcagagcgcggcgc

African Green monkey HSP70
Sainis,I., Angelidis,C., Pagoulatos,G. and Lazaridis,I.
FEBS Lett. 355 (3), 282-286 (1994)

Gaattccgtttctagagcgtggctcccgttgtcccgaggcttcccagagcgaacctgtgcggctgcaggcaccagcg
ccgttgagtttccggcgttccggaggactgagctcttgtcacgggtcccgtccgccgtttccagtcccgaatctcggagc
ggacgagacagcagggcaccggc

Bos taurus Angus HSP70
ACCESSION U02892
NID g414974
AUTHORS Grosz,M.D. and Skow,L.C.(unpublished)

gccgcctgaggagaaacagcagcctggagagagctgataaaacttacggcttagtccgtgagagcagttccgcag
acccgctatctccaaggaccgcgaggggcaccagagcgttcagttttcgggttccgaaaagcccgagcttctcgtcg
cagatcctcttcaccgatttcagtttgaagcttatttcggagccgaaaaagcagggcaccgc

This sequence is only available in Genbank data base (g414974).

Below, for reference purposes, are shown the sequence identity of the human hsp70A 5'UTR with the hsp70 5'UTR from various other species and with the human HSP70B 5'UTR :

var/tmpweb/analseq/al9179/s1 : 215 nt
ALIGN calculates a global alignment of two sequences
version 2.0uPlease cite: Myers and Miller, CABIOS (1989) 4:11-17
human 215 nt vs.

rat 217 nt

scoring matrix: DNA, gap penalties: -16/-4

49.2% identity; Global alignment score: -102

resetting matrix to DNA

/var/tmpweb/analseq/al10142/s1 : 215nt

ALIGN calculates a global alignment of two sequences

version 2.0uPlease cite: Myers and Miller, CABIOS (1989) 4:11-17

human 215 nt vs.

chicken 111 nt

scoring matrix: DNA, gap penalties: -16/4

32.7% identity; Global alignment score: -349

resetting matrix to DNA

/var/tmpweb/analseq/al10710/s1 : 215 nt

ALIGN calculates a global alignment of two sequences

version 2.0uPlease cite: Myers and Miller, CABIOS (1989) 4:11-17

human 215 nt vs.

mouse 218 nt

scoring matrix: DNA, gap penalties: -16/-4

49.6% identity; Global alignment score: -39

resetting matrix to DNA

/var/tmpweb/analseq/al10998/s1 : 215 nt

ALIGN calculates a global alignment of two sequences

version 2.0uPlease cite: Myers and Miller, CABIOS (1989) 4:11-17

human 215 nt vs.

green monkey 180 nt

scoring matrix: DNA, gap penalties: -16/-4

69.3% identity; Global alignment score: 403

## Examples

*Materials*

*Cell culture medium (BME and MEM), penicillin, streptomycin, trypsin-EDTA solution, versene , non-essential amino acids and restriction enzymes were obtained from Gibco, Life Technologies, Inc. Foetal bovine serum (heat inactivated) was from HyClone, Culture flasks (TPP T150) and 60mm culture dishes (Falcon) were purchased from Becton Dickson. Plasmids were obtained from Promega. The HepG2, Hep3B, HEK293 and WI-38 cell lines were obtained from the American Type Culture Collection.*

Methods

***Plasmids constructs***

HSP70 reporter vectors were generated using pGL3 promoter vector plasmid for the firefly luciferase assay, or pCAT3 promoter vector plasmid for the chloramphenicol acetyl transferase (CAT ) assay were purchased from PROMEGA. Human HSP70 promoter ,human HSP70 5'UTR and 3'UTR were PCR-amplified (Advantage GC genomic PCR kit, Clontech) from a human genomic bank (Clontech). Oligonucleotides were designed from the human hsp70A gene (Hunt C. and Morimoto R.I.(1985) Proc.Natl.Acad.Sci.USA 82, 6455-6459) (GenBank : g184416).

Sequence of the human Hsp70 5'UTR and regions (bold characters) utilized to design oligonucleotides for the PCR reaction :

5'-

**ataacggctagcctgagg**agctgctgcgacagtccactacctttttcgagagtgactcccgttgtcccaaggcttcc cagagcgaacctgtgcggctgcaggcaccggcgcgtcgagtttccggcgtccggaaggaccgagctcttctcgcgg atccagtgttccgtttccagcccccaatctcagagccgagccga **agagagcagggaaccgc**-3'

For the the human Grp78 5'UTR primers were designed using the sequence published by Ting J. and Lee A.S. (1988) DNA (4) 275-278 (GenBank : g183644).

The 5'UTRs were inserted between the Hind III and Nco I sites, HSP70 promoter between Bgl II and Nco I sites. The human Hsp70 3'UTR was inserted between the Xbal and BamH1 sites.

Dicistronic constructs were generated using pCI-neo (PROMEGA). The Hsp705'UTR-luciferase or the FMDV-IRES-luciferase were cloned in the EcoR I site.

All the constructs were sequence-checked.

*Cell culture conditions*

HepG2 and WI-38 cell lines were maintained in BME supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin-glutamine. For HepG2 cells, medium was supplemented with 1% non essential amino acids, 1% sodium. Hep3B and HEK293 were maintained in MEM (Gibco Life Technologies, Inc) supplemented with 10% fetal bovine serum (HyClone) and 1% penicillin-streptomycin-glutamine. Cells were maintained at 37°C in humidified air containing 5% $CO_2$.

*Transient transfections*

HepG2, Hep3B, 293 and WI-38 cells were transiently transfected with the indicated construct and the internal control pRL-TK vector for luciferase assay or pSVe-βGal vector for CAT assay, using the calcium phosphate co-precipitation method.

*Quantification of reporter genes activities*

Reporter gene activities were quantify 48 hours after transfection. In heat-shock experiments, 48h hours after transfection the cells were heat-shocked at 42°C for 40 minutes and then maintained 4 hours at 37°C before luciferase activities were measured.

Luciferase activities were quantified using the Dual Luciferase Assay (Promega). Values were normalised with the renilla luciferase activity expressed from pRL-TK. CAT activities were normalised with the β galactosidase activity expressed from pSVe-βGal .

*CAT mRNA quantification*

The $^{33}P$ CAT probe was synthetized with linearized pTRI-CAT vector (CAT Direct™: CAT mRNA detection kit, Ambion) using MaxiScript™ : *in vitro* transcription kit (Ambion), according to the manufacturer's protocol.

Confluent transfected cells were washed with phosphate-buffered saline (PBS). Cells were lysed in TRIZOL (Gibco, Life Technologies, Inc). Total mRNA was extracted using a 24:1 v/v of chloroform/isoamyl alcohol. Total mRNA was pelleted with an equal volume of isopropanol. Pellet was washed with a cold 70% ethanol solution and then solubilized in lysis buffer of Direct Protect™ kit (Lysate Ribonuclease Protection Assay, Ambion).

CAT mRNAs were quantified by a lysate ribonuclease protection assay using Direct Protect™ kit (Ambion) according to the manufacturer's protocol.

Protected fragments were resolved in 5% poly-acrylamide gels containing 8M urea and radioactivity was quantified using a Phosphorimager (STORM, Molecular Dynamics).

Example 1: Effect of the 5'UTR of the human HSP70 mRNA on the expression of a luciferase reporter driven by the HSP70 promoter (Figure 1).

The human HSP70 promoter was cloned upstream of the coding sequence of the firefly luciferase gene in the absence or presence of the 5' UTR (plasmids A and B respectively). HepG2 cells were transfected with these two chimeric constructs and the levels of luciferase were compared either in normal conditions or after a 30 min heat-shock at 42°C. The presence of the 5'UTR of the human HSP70 mRNA by itself strongly increased the level of expression of the luciferase. Under both conditions (normal and heat-shock) a similar 9-fold stimulation was observed in the presence of the 5'UTR demonstrating that this increase in the luciferase expression is inherent to this 5'UTR and independent of the stress. Therefore, it can be expected that the human Hsp70 5'UTR can be used to enhance the expression of a wide variety of genes in normal cellular conditions.

Example 2: Effect of the 5'UTR of the human HSP70 mRNA on the expression of luciferase in various cell lines (Figure 2).

HSPs are ubiquitous proteins and the heat-shock response is a highly conserved mechanism. Therefore, a similar effect of the human Hsp70 5'UTR was expected in other cell lines. Three other human cell lines were transfected with the same constructs. Hep3B is a hepatic cell line very close to HepG2. The two other cell lines tested, WI-38 and HEK293, are from different origins. WI-38 is a fibroblast-like cell line derived from embryonic lung tissue and HEK293 is a transformed primary embryonal kidney cell line. As shown in Figure 2, the 5'UTR of the human HSP70 mRNA increased the expression of the luciferase gene in the three cell lines tested. An ~9-fold stimulation was obtained in the presence of the 5'UTR in Hep3B cells comparable to the stimulation observed in HepG2. In WI-38 and HEK293 cells the effect of the 5'UTR was less (a ~ 5-fold stimulation) but still significant, showing that this 5'UTR effect is not cell type specific. Therefore, it can be expected that this sequence can be used in a broad spectrum of applications where genes are expressed in various cell.

Example 3: Effect of the human HSP70 5'UTR on the translational efficiency of the chloramphenicol- acetyl-transferase mRNA (Figure 3).

A higher level of luciferase expression in the presence of the HSP70 5'UTR can be explained either by a higher level of luciferase mRNA (due to an increase either in transcription or in mRNA stability) or by a more efficient translation of the luciferase mRNA. In order to distinguish between these two possible mechanisms, the mRNA levels in the absence or in the presence of the HSP70 5'UTR (plasmid H and I, respectively) were measured in transfected HepG2 cells. In this experiment CAT gene was used as reporter and was driven by the SV40. As previously observed with the luciferase gene, the presence of the HSP70 5'UTR cloned upstream of the coding sequence of the CAT gene increased (~10-fold) the CAT activity. Moreover, this higher level of CAT was achieved without any significant change in the CAT mRNA level in the presence of the HSP70 5'UTR. This result shows that the HSP70 5'UTR increases the translational efficiency of the mRNA independently of the reporter gene or the promoter used. Therefore, it can be expected that this human Hsp70 5'UTR property of increasing translational efficiency can be obtained for a wide variety of genes and promoters and can thus be used for a broad range of applications.

Example 4: Effect of human Hsp70 5'UTR on the expression of the luciferase in presence of the SV40 3'UTR (Figure 4).

The 3'UTR present downstream of the reporter coding sequence in commercially available plasmids is frequently the 3'UTR of the SV40 large T antigen. This viral 3'UTR is known to allow a high level of expression of the reporter in transfection experiments. In order to determine if the human HSP70 5'UTR can increase the expression of the luciferase with this heterologous SV40 3'UTR we generated two vectors containing the SV40 3'UTR in the presence or absence of the human HSP70 5'UTR (plasmid C and D respectively). As expected (Figure 4) the SV40 large T antigen 3'UTR allowed a high level of expression of the luciferase gene in the three cell lines tested. A 5- to 10-fold higher level of luciferase was observed in presence

of this viral 3'UTR in comparison to the levels of expression obtained with the human HSP70 3'UTR (Figures 1 and 2). Nevertheless, the presence of the human HSP70 5'UTR was still able to increase the level of the luciferase expression by a 2-fold factor in the three cell lines tested.

Example 5: The effect on translation is not a common property of all stress protein 5'UTR (Figure 5).

In order to compare the effect of the HSP70 5'UTR with another stress protein 5'UTR, the human GRP78 5'UTR was used. The human GRP78 5'UTR has a similar length as the HSP70 5'UTR (221bp versus 215pb for HSP70), and both of them are equally G + C rich (63%). Using the MFOLD program (Zuker M. and Jacobson A.B. (1995) Nucleic Ac. Res. (23) 2791-2798) to calculate the stability of these two 5'UTRs, a similar high □G value was found (~ -60 kCal/mol), suggesting that these two 5'UTRs form structures of comparably high stability. Therefore the GRP78 5'UTR was an interesting 5'UTR to compare to the HSP70 5'UTR. As shown in Figure 5, this 5'UTR (plasmid G) does not modify the level of expression of the luciferase gene. This result shows that the effect obtained with the HSP70 5'UTR is not a common property of all stress protein 5'UTRs.

Example 6: The Hsp70 5'UTR does not behave as an Internal Ribosomal Entry Site (IRES) (Figure 6).

IRES stuctures are found in the 5'UTR region of picornaviral mRNAs as well as in few eukaryotic mRNAs (Sachs A.B. *et al* (1997) Cell (89) 831-838). These structures allow a cap-independent protein translation. The decisive experiment to reveal an IRES structure is the use of a dicistronic plasmid with the putative IRES cloned between two Open-Reading-Frames (ORFs) under the control of one unique promoter. When the sequence contains an IRES structure the second ORF is translated independently of the presence of an upstream first ORF. Such plasmids were obtained with either the Human Hsp70 5'UTR (plasmid J) or with a classical IRES structure ( the Foot and Mouth Disease Virus IRES (FMDV-IRES)) (plasmid K)

or without any sequence (plasmide L) between the first ORF and the luciferase gene (second ORF). As shown in Figure 6, the FMDV-IRES is capable of initiating the translation of the luciferase in a dicistronic context. In contrast no luciferase activity is obtained either with the Human Hsp70 5'UTR or without any sequence upstream of the luciferase coding sequence. This result shows that the Human Hsp70 5'UTR does not contain an IRES structure.

Example 7: Effect of HSP70 element on expression of ovalbumin protein in vitro

To determine whether the translation-enhancing properties of the HSP70 5' UTR element would be advantageous in DNA vaccination, we constructed a series of expression plasmids for the model antigen chicken ovalbumin. The plasmids all incorporate a common backbone derived from the vector pCI (Promega, Southampton, UK). Key elements in this plasmid are the immediate early promoter from human cytomegalovirus, which drives expression of the inserted antigen, a SV40 polyadenylation and transcription terminator sequence and the ampicillin resistance gene.

A cDNA cassette encoding the entire coding region of the chicken ovalbumin gene (GenBank accession V00383) was inserted into the plasmid between the promoter and SV40 element, creating pOvaREP. A second variant was prepared (pOvaOLD), containing an ATG sequence upstream from the ovalbumin gene translation initiation codon. This alteration markedly suppresses translation efficiency in transcripts from pOvaOLD compared with pOvaREP. The 5'UTR element of HSP70 was cloned into both vectors, creating pHSPOvaREP and pHSPOvaOLD.

All four constructs were sequence validated before being transiently transfected into four different cell lines (HepG2, 293, HeLa and CHO cells) to evaluate expression levels. To allow correction for transfection efficiency, a fixed amount

of a luciferase expression plasmid (pGL3-Control; Promega) was also included in each transfection. Culture supernatant was collected 48 hours (CHECK) after transfection, and assayed for luciferase activity and ovalbumin content by ELISA.

The ovalbumin production in each sample was normalised for transfection efficiency using the luciferase activities. Relative protein expression levels are represented as a ratio of levels seen with and without the HSP70 element (see Table 1 below). No meaningful results were obtained for the pOvaOLD and pHSPOvaOLD constructs as the levels of ovalbumin produced were beneath the detection limit of the assay. However, pHSPOvaREP produced significantly more ovalbumin than pOvaREP in all four cell lines. The effect of the HSP70 element was most marked in the 293 cell line.

**Table 1**. Effect of HSP70 element on ovalbumin production from pOva plasmids in vitro.

| ***Cell type*** | ***Ratio Ova expression pHSPOvaREP: pOvaREP*** |
|---|---|
| *CHO* | 1.5 |
| *HeLa* | 1.7 |
| *Hep G2* | 1.9 |
| *293* | 3.0 |

Data are the means of triplicate determinations.

Example 2: Immunisation of mice with ovalbumin expression plasmids containing HSP70 5'UTR element.

The four plasmids described in Example 1 were used to vaccinate female Balb/c mice by biolistic gene delivery (REF – PJV patents). Animals were divided into groups of 6 and given two shots of 0.5ug of plasmid at day 0 and again at day 43. Serum samples were taken at day -1, day 21, day 42, day 57 and day 71. Specific anti-ovalbumin IgG titres were determined by ELISA.

All four plasmids induced a similar response to the priming dose, with no apparent differences between groups at the day 21 and day 42 time points. However, following the day 43 immunisation, significant differences emerged (Table 2). Inclusion of the HSP70 5'UTR element enhanced the immune response to both the poor-expressing pOvaOLD and the optimally-expressing pOvaREP. These data suggest that the HSP70 5'UTR element is useful in enhancing the efficacy of DNA vaccination.

**Table 2.** Anti-ovalbumin IgG titres in vaccinated mice

| *Plasmid* | *Reciprocal titre* |
|---|---|
| pOvaOLD | 1100 |
| pHSPOvaOLD | 1500 |
| pOvaREP | 7000 |
| pHSPOvaREP | 10000 |

Anti Ova antibody titres were determined at the half-maximal absorbance value. Bleeds were from 57 days post primary immunisation.

The present invention thus also provides a DNA molecule according to the invention for use in therapy, preferably in therapeutic or prophylactic vaccination, for example when administered by particle bombardment, most preferably for use in achieving an increased immune response.

The present invention further provides a method of therapeutic or prophylactic vaccination comprising administering an effective amount of a DNA molecule according to the invention. Preferably the DNA molecule is administered by particle bombardment, most preferably for use in achieving an increased immune response.

**Definitions**

For the avoidance of doubt, certain terms used herein are further defined below. Similar terms should be construed accordingly.

**"Polypeptide"**

This means any moiety having a plurality of amino-acids joined together by peptide bonds. It includes proteins and peptides.

**"About"**

When used in connection with a numerical value this term allows for a margin either side of the value. Preferably the margin is +/- 10 % of the figure. more preferably it is +/- 5 %

**"Sequence identity"**

For the purposes of the present invention, sequence identity may be determined, for example, by using the ALIGN program (version 2.0). This calculates a global alignment of two sequences. (See Myers and Miller,(1989) CABIOS, **4**, 11-17). Gap penalties: -16/-4. For information see http://www.infobiogen.fr/services/menuserv.html

**"Substantial Sequence identity"**

This term is used to include polynucleotide sequences having at least 50% sequence identity with a given polynucleotide sequence. Preferably the degree of sequence

identity is at least 75%. Sequence identities of at least 90%, at least 95% or at least 99% are most preferred.

**"Heat Shock"**

This is an increase in temperature which is sufficient to induce the heat shock response. Classically for cells a shift from 37°C to 42°C for 30 minutes is used to induce a heat shock.

**"Increased Efficiency of Translation"**

This means that a greater degree of translation to provide active polypeptides is obtained from a given number of mRNA molecules than would otherwise be the case.

**Remarks**

The foregoing description of the invention is merely illustrative thereof and it should therefore be apprecaited that various variations and modifications can be made without departing from the spirit or scope of the invention as set forth in the accompanying claims.

Where preferred or optional features are described in connection with particular aspects of the present invention, they shall be deemed to apply *mutatis mutandis* to othe aspects of the invention unless the context indicates otherwise.

All documents cited herein are hereby incorporated by reference, as are any citations referred to in said documents.

## Claims

1. A DNA molecule that can be transcribed to provide an RNA molecule having an untranslated region that provides an increased efficiency of translation of a polypeptide when operably linked to a region encoding said polypeptide; wherein said DNA molecule

(i) does not encode a mammalian Hsp70;

(ii) does not comprise an hsp promoter; and

(iii) comprises

a) the sequence:
5'ataacggctagcctgaggagctgctgcgacagtccactacctttttcgagagtgactcccgttgtcccaaggcttccc
agagcgaacctgtgcggctgcaggcaccggcgcgtcgagtttccggcgtccggaaggaccgagctcttctcgcgg
atccagtgttccgtttccagcccccaatctcagagccgagccgacagagagcagggaaccgc-3',

b) the complement of the sequence given in a), or

c) a sequence having substantial sequence identity with a sequence as defined in a) or b) above.

2. A DNA molecule according to claim 1; wherein said untranslated region is a 5' untranslated region.

3. A DNA molecule according to claim 1 or 2 wherein said untranslated region has a $\Delta G$ of below -10 kCal/mol.

4. A DNA molecule according to any preceding claim wherein said sequence has a $\Delta G$ that is below -30 kCal/Mol.

5. A DNA molecule according to any preceding claim wherein said sequence has a ΔG that is below -40 kCal/Mol.

6. A DNA molecule according to any preceding claim wherein said untranslated region has a ΔG of below -50 kCal/Mol.

7. A DNA molecule according to any preceding claim wherein expression of said polypeptide is heat shock responsive.

8. An RNA molecule obtainable by transcribing a DNA molecule according to any of claims 1 to 7.

9. A vector comprising a DNA molecule according to any of claims 1 to 7.

10. An expression system comprising a DNA molecule according to any of claims 1 to 7 or a vector according to claim 9.

11. An expression system according to claim 10 which comprises one or more cells.

12. An expression system according to claim 11 comprising one or more eukaryotic cells.

13. An expression system according to claim 11 comprising one or more mammalian cells.

14. An expression system according to claim 11 comprising one or more human cells.

15. An expression system according to claim 10 which is a cell free expression system.

16. A method of obtaining a polypeptide comprising expressing the polypeptide using an expression system according to any of claims 10 to 15 and, optionally, purifying the polypeptide.

17. A method according to claim 16 comprising the step of providing the expression system with a heat shock.

18. A method of treating a deficiency in the expression of a polypeptide, comprising providing a patient with a DNA molecule as claimed in any of claims 1 to 7 which encodes said polypeptide, a vector as claimed in claim 9 comprising said DNA molecule, or a cell comprising said DNA molecule or vector.

19. A method of treating a deficiency in the expression of a polypeptide, comprising providing a patient with a DNA molecule as claimed in any one of claims 1 to 7 wherein said molecule is provided in a manner to allow it to become operably linked with a sequence already present in the patient which encodes said polypeptide.

20. A method of treating a disorder (e.g. an infection) treatable by providing an increased immune response, comprising providing a patient with a vaccine comprising a DNA molecule as claimed in any of claims 1 to 7 or a vector as claimed in claim 9.

21. A method according to claim 18 or 19; wherein a DNA molecule or vector is provided under conditions allowing it to integrate within the patient's genome.

22. A method according to claim 18, wherein a cell is provided under conditions allowing it to be maintained within the patient.

23. A method according to claim 22 wherein said cell is a cell that has been removed from the patient and has been modified prior to being reintroduced to the patient.

24. A method of treating a deficiency in the expression of a polypeptide, comprising providing the patient with an RNA molecule as claimed in claim 8.

25. A pharmaceutically acceptable composition comprising a DNA molecule according to any of claims 1 to 7, an RNA molecule according to claim 8, or a cell as described in any of claims 11 to 14.

26. A vaccine comprising a DNA molecule according to any of claims 1 to 5, or a vector according to claim 9.

27. The use of a DNA molecule according to any of claims 1 to 7, of an RNA molecule according to claim 8, of a vector according to claim 9, or of an expression system according to any of claims 10 to 15, in achieving increased expression of a polypeptide.

28. A DNA molecule according to any one of claims 1 to 7 for use in therapy.

29. A DNA molecule according to claim 28 for use in therapeutic or prophylactic vaccination.

30. A DNA molecule according to claim 28 or 29 when administered by particle bombardment.

31. A DNA molecule according to claim 28, 29 or 30 for use in achieving an increased immune response.

32. Method of therapeutic or prophylactic vaccination comprising administering an effective amount of a DNA molecule as claimed in any one of claims 1 to 7.

33. Method according to claim 32 wherein the DNA molecule is administered by particle bombardment.

34. Method according to claim 32 or 33 for use in achieving an increased immune response.

**Figure 1**

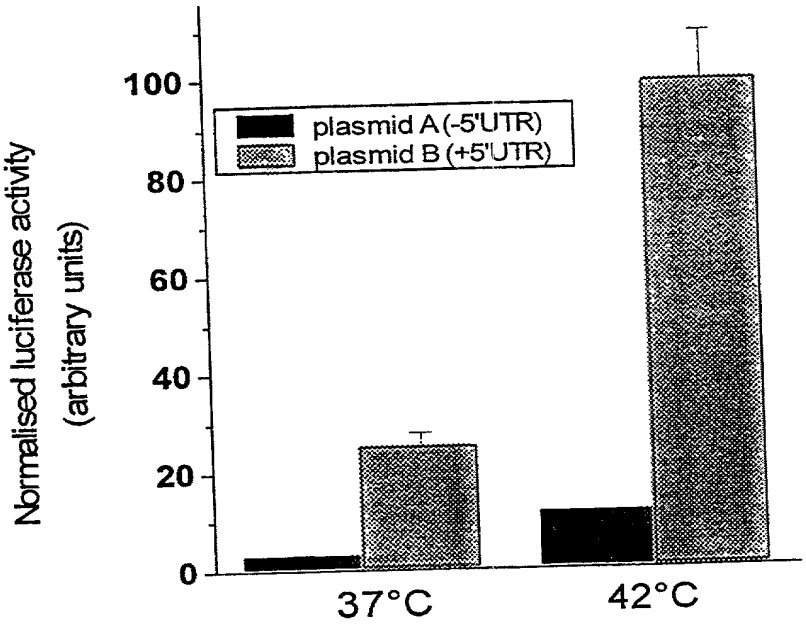

Normalised luciferase activity
(arbitrary units)
100
80
60
40
20
0
plasmid A (-5'UTR)
plasmid B (+5'UTR)
37°C
42°C

**Figure 2**

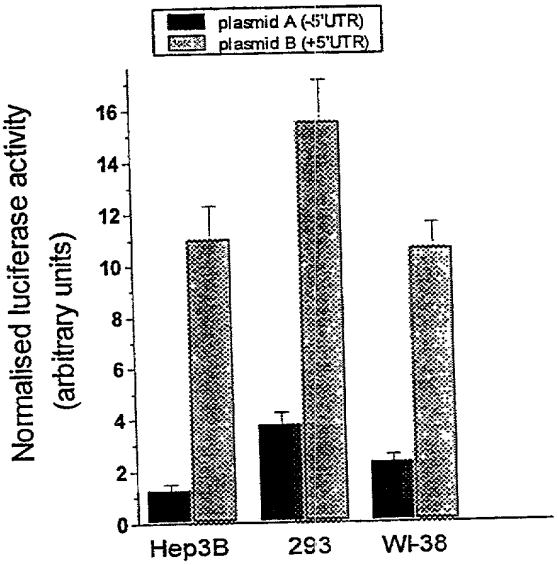

plasmid A (-5'UTR)
plasmid B (+5'UTR)
Normalised luciferase activity
(arbitrary units)
0
2
4
6
8
10
12
14
16
Hep3B
293
WI-38

**Figure 3**

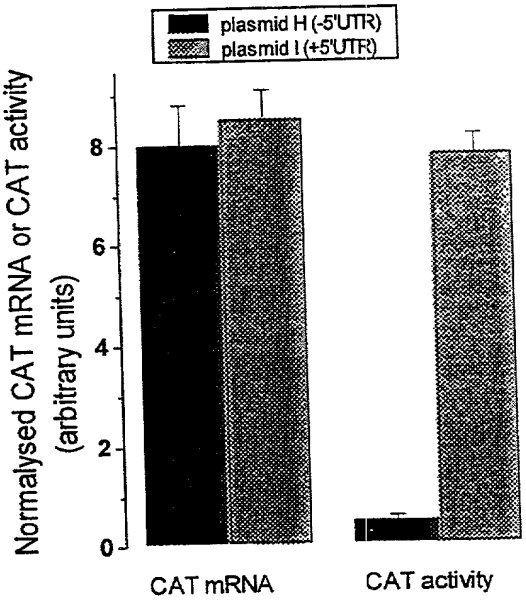

plasmid H (-5'UTR)
plasmid I (+5'UTR)
Normalysed CAT mRNA or CAT activity (arbitrary units)
0
2
4
6
8
CAT mRNA
CAT activity

**Figure 4**

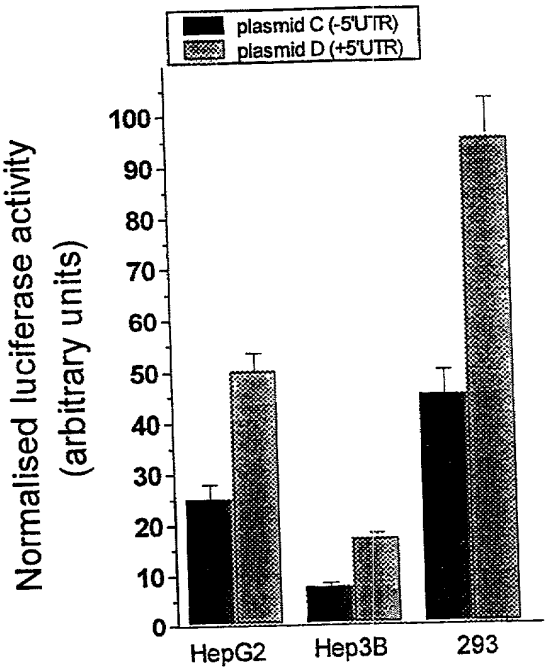

plasmid C (-5'UTR)
plasmid D (+5'UTR)
Normalised luciferase activity
(arbitrary units)
0
10
20
30
40
50
60
70
80
90
100
HepG2
Hep3B
293

**Figure 5**

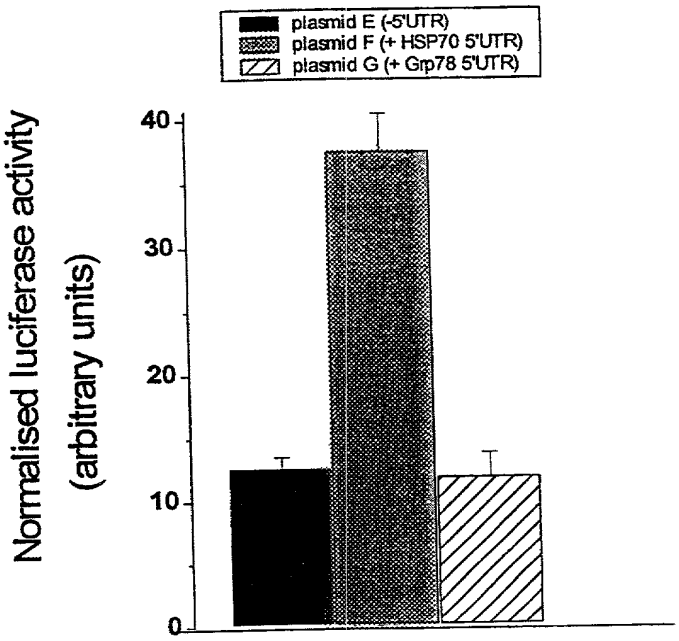

plasmid E (-5'UTR)
plasmid F (+ HSP70 5'UTR)
plasmid G (+ Grp78 5'UTR)
Normalised luciferase activity
(arbitrary units)
40
30
20
10
0

**Figure 6**

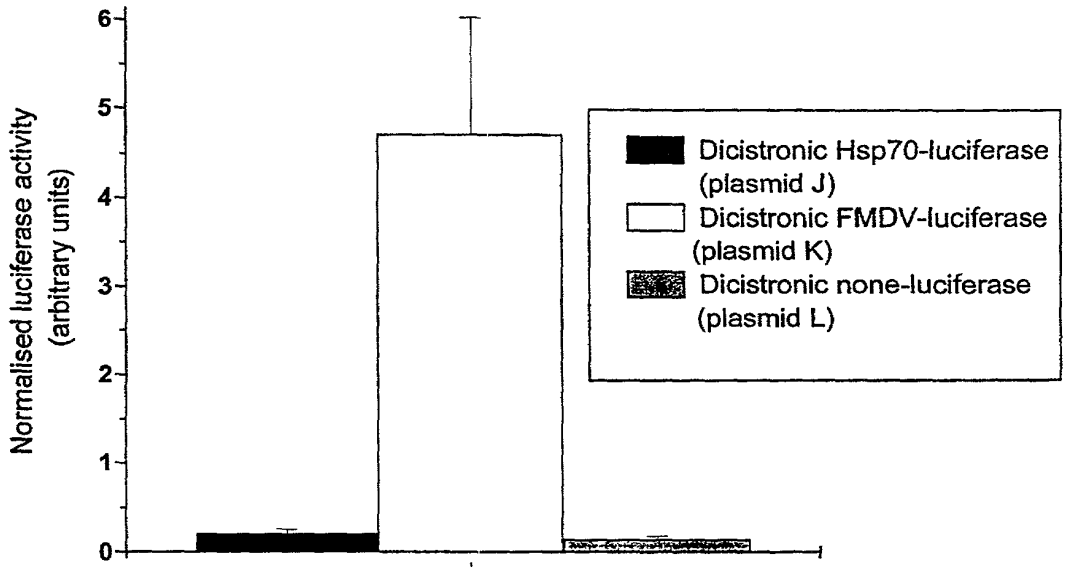
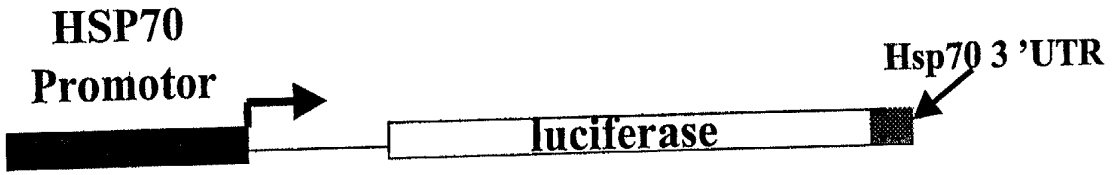
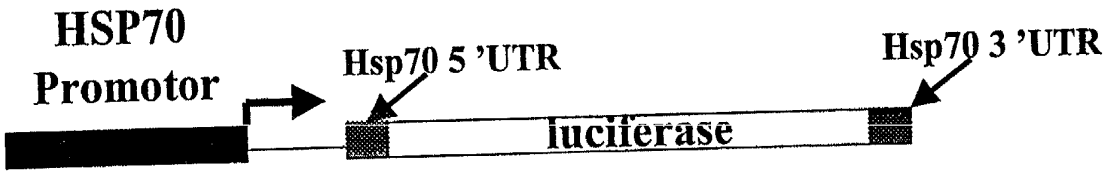

Normalised luciferase activity
(arbitrary units)
0
1
2
3
4
5
6
Dicistronic Hsp70-luciferase
(plasmid J)
Dicistronic FMDV-luciferase
(plasmid K)
Dicistronic none-luciferase
(plasmid L)

Figure 7

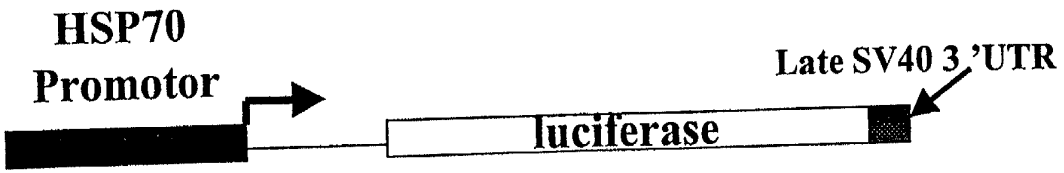
Plasmid A




Plasmid B

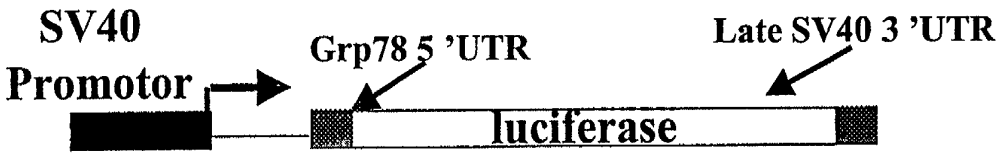



Plasmid C

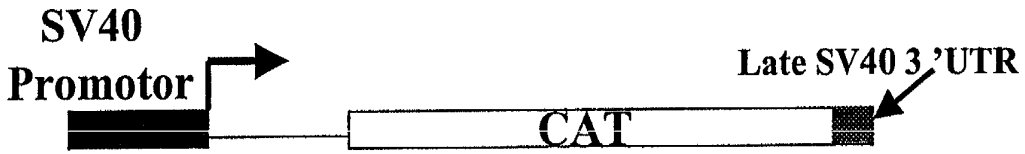


HSP70
Promotor
Hsp70 5 'UTR
luciferase
Late SV40 3 'UTR
Plasmid D
SV40
Promotor
luciferase
Late SV40 3 'UTR
Plasmid E
(pGL3pv)
SV40
Promotor
Hsp70 5 'UTR
luciferase
Late SV40 3 'UTR
Plasmid F

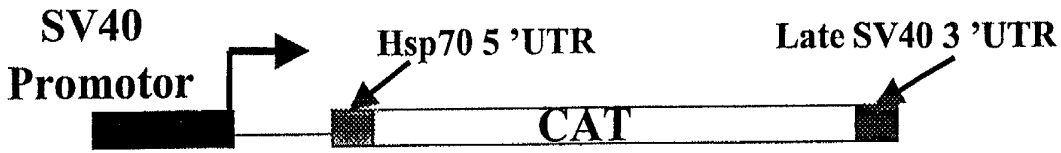
Plasmid G



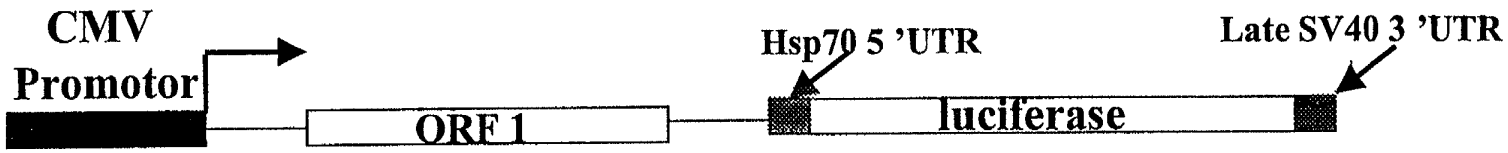

Plasmid H



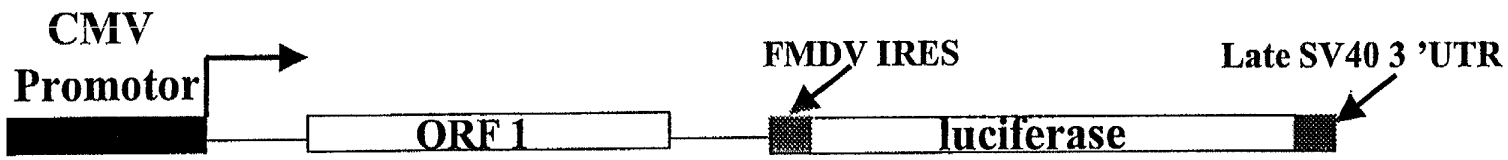

Plasmid I



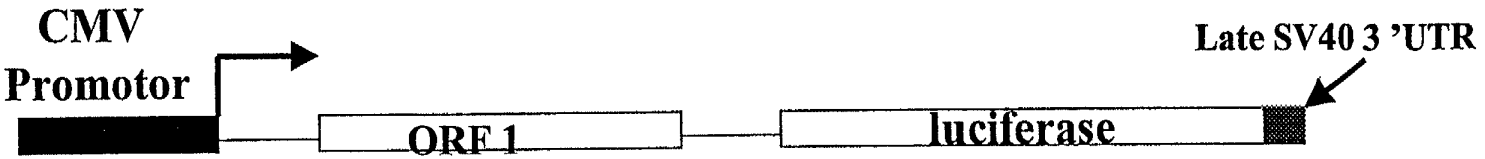
## Plasmid J




## Plasmid K




## Plasmid L



# COMBINED DECLARATION FOR UTILITY OR DESIGN PATENT APPLICATION WITH POWER OF ATTORNEY

| ATTORNEY'S DOCKET |
|---|
| PF3623USW |
| First Names Inventor. |
| **COSTE** |
| ***Complete if known:*** |
| App No.: |
| Filing Date |
| September 11, 2001 |
| Group Art Unit: |

( ) Declaration submitted with initial filing or

( )Declaration submitted after initial filing (surcharge required 37CFR1.16(e))

As below named inventor. I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

**EXPRESSION**

the specification of which (check only one item below):

[ ]is attached hereto.
OR
[X] was filed on _____as United States application Serial No. _____ or **PCT** International

Application Number PCT/EP00/02031 filed March 9, 2000 and was amended on (MM/DD/YYYY) _____(if applicable)

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment specifically referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR §1.56.

I hereby claim foreign priority benefits under 35, U.S.C. §119 (a)-(d) or §365(b) of any foreign applications(s) for patent or inventor's certificate or 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate or of any PCT international application having a filing date before that of the application on which priority is claimed:

| Prior Foreign Application Number (s) | Country | Foreign Filing Date (MM/DD/YYYY)) | PRIORITY CLAIMED |
|---|---|---|---|
| 1. GB9905498.3 | GB | 03/11/1999 | x |
| 2. | | | |
| 3. | | | |
| 4. | | | |
| 5. | | | |

I hereby claim the benefit under Title 35, United States Code §119(e) of any United States provisional application(s) listed below:

| Application No. | Filing Date (MM/DD/YYYY) | |
|---|---|---|
| | | |
| 2. | | |
| 3. | | |
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# COMBINED DECLARATION FOR UTILITY or DESIGN PATENT APPLICATION WITH POWER OF ATTORNEY Continued

ATTORNEY'S DOCKET NUMBER: PF3623USW

I hereby claim the benefit under 35, U.S.C. §120 of any United States application or §365(c) of any PCT international application designating the United States of America that is listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. §112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 C.F.R. §1.56 which became available between the filing date of the prior application(s) and the national or PCT international filing date of this application:

## PRIOR U.S. PARENT APPLICATION or PCT PARENT APPLICATION

| U.S. Parent Application or PCT Parent Number | Parent Filing Date (MM/DD/YYYY) | STATUS (Check one) PATENTED | PENDING | ABANDONED |
|---|---|---|---|---|
| | | | | |
| | | | | |
| | | | | |

**POWER OF ATTORNEY:** As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the U.S. Patent and Trademark Office connected therewith. (List name and registration number)

| | | | | | |
|---|---|---|---|---|---|
| David J. Levy | Reg. No. 27,655 | James P. Riek | Reg. No. 39,009 | Bonnie L. Deppenbrock | Reg. No. 28,209 |
| Charles E. Dadswell | Reg. No. 35,851 | Virginia C. Bennett | Reg. No. 37,092 | John L. Lemanowicz | Reg. No. 37,380 |
| Karen L. Prus | Reg. No. 39,337 | Frank P.Grassler | Reg. No. 31,164 | Amy H. Fix | Reg No 42,616 |
| Robert H. Brink | Reg. No. 36,094 | Christopher P. Rogers | Reg. No. 36,334 | | |
| Elizabeth Selby | Reg No. 38,298 | Lorie Ann Morgan | Reg. No. 38,181 | | |

Send Correspondence to:
**David J. Levy, Patent Counsel**
**Corporate Intellectual Property Department**
**GlaxoSmithKline,**
**Five Moore Drive, PO Box 13398**
**Research Triangle Park, NC 27709**

Direct Telephone Calls to:
Elizabeth Selby
919-483-3934

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

| | | | | | |
|---|---|---|---|---|---|
| 2 | FULL NAME OF INVENTOR | FAMILY NAME: **COSTE** | FIRST GIVEN NAME: **Herve** | SECOND GIVEN NAME/INITIAL: **Jean-Clement** | |
| | **INVENTOR'S SIGNATURE** | | | DATE: 11/12/2001 | |
| 0 | RESIDENCE & CITIZENSHIP | CITY: **Les Ulis** FRX | STATE OR FOREIGN COUNTRY: **Essonne, FR** | COUNTRY OF CITIZENSHIP: **FR** | |
| 1 | POST OFFICE ADDRESS | POST OFFICE ADDRESS: **GlaxoSmithKline Five Moore Drive, PO Box 13398** | CITY: **Research Triangle Park** | STATE & ZIP CODE/COUNTRY: **NC 27709 US** | |
| 2 | FULL NAME OF INVENTOR | FAMILY NAME: **ELLIS** | FIRST GIVEN NAME: **Jonathan** | SECOND GIVEN NAME/INITIAL: **Henry** | |
| | **INVENTOR'S SIGNATURE** | | | DATE: | |
| 0 | RESIDENCE & CITIZENSHIP | CITY: **Stevenage** GBX | STATE OR FOREIGN COUNTRY: **Hertfordshire, GB** | COUNTRY OF CITIZENSHIP: **GB** | |
| 2 | POST OFFICE ADDRESS | POST OFFICE ADDRESS: **GlaxoSmithKline, Inc. Five Moore Drive, PO Box 13398** | CITY: **Research Triangle Park** | STATE & ZIP CODE/COUNTRY: **NC 27709 US** | |

# COMBINED DECLARATION FOR UTILITY OR DESIGN PATENT APPLICATION WITH POWER OF ATTORNEY

| ATTORNEY'S DOCKET PF3623USW |
|---|
| First Names Inventor: **COSTE** |
| ***Complete if known:*** App No.: |
| Filing Date September 11, 2001 |
| Group Art Unit: |

( ) Declaration submitted with initial filing or

( )Declaration submitted after initial filing (surcharge required 37CFR1.16(e))

As below named inventor. I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

**EXPRESSION**

the specification of which (check only one item below):

[ ]is attached hereto.
OR
[X] was filed on _____as United States application Serial No. _____ or **PCT** International

Application Number PCT/EP00/02031 filed March 9, 2000 and was amended on (MM/DD/YYYY) _____(if applicable)

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|---|---|---|---|
| 1. GB9905498.3 | GB | 03/11/1999 | x |
| 2. | | | |
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I hereby claim the benefit under Title 35, United States Code §119(e) of any United States provisional application(s) listed below:

| Application No. | Filing Date (MM/DD/YYYY) | |
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| 2. | | |
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# COMBINED DECLARATION FOR UTILITY or DESIGN PATENT APPLICATION WITH POWER OF ATTORNEY Continued

ATTORNEY'S DOCKET NUMBER
**PF3623USW**

I hereby claim the benefit under 35, U.S.C. §120 of any United States application or §365(c) of any PCT international application designating the United States of America that is listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. §112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 C.F.R. §1.56 which became available between the filing date of the prior application(s) and the national or PCT international filing date of this application:

**PRIOR U.S. PARENT APPLICATION or PCT PARENT APPLICATION**

| U.S. Parent Application or PCT Parent Number | Parent Filing Date (MM/DD/YYYY) | STATUS (Check one) PATENTED | PENDING | ABANDONED |
|---|---|---|---|---|
| | | | | |
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| | | | | |

**POWER OF ATTORNEY:** As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the U.S. Patent and Trademark Office connected therewith. (List name and registration number)

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| Charles E. Dadswell | Reg. No. 35,851 | Virginia C. Bennett | Reg. No. 37,092 | John L. Lemanowicz | Reg. No 37,380 |
| Karen L. Prus | Reg. No. 39,337 | Frank P.Grassler | Reg. No. 31,164 | Amy H. Fix | Reg. No. 42,616 |
| Robert H. Brink | Reg. No 36,094 | Christopher P. Rogers | Reg. No. 36,334 | | |
| Elizabeth Selby | Reg. No. 38,298 | Lorie Ann Morgan | Reg. No. 38,181 | | |

Send Correspondence to:
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**Corporate Intellectual Property Department**
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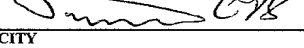
| 2 | FULL NAME OF INVENTOR | FAMILY NAME **COSTE** | FIRST GIVEN NAME **Herve** | SECOND GIVEN NAME/INITIAL **Jean-Clement** |
| | **INVENTOR'S SIGNATURE** | | | DATE: |
| 0 | RESIDENCE & CITIZENSHIP | CITY **Les Ulis** | STATE OR FOREIGN COUNTRY **Essonne, FR** | COUNTRY OF CITIZENSHIP **FR** |
| 1 | POST OFFICE ADDRESS | POST OFFICE ADDRESS **GlaxoSmithKline Five Moore Drive, PO Box 13398** | CITY **Research Triangle Park** | STATE & ZIP CODE/COUNTRY **NC 27709 US** |
| 2 | FULL NAME OF INVENTOR | FAMILY NAME **ELLIS** | FIRST GIVEN NAME **Jonathan** | SECOND GIVEN NAME/INITIAL **Henry** |
| | **INVENTOR'S SIGNATURE** |  | | DATE: 11 December 2001 |
| 0 | RESIDENCE & CITIZENSHIP | CITY **Stevenage** | STATE OR FOREIGN COUNTRY **Hertfordshire, GB** | COUNTRY OF CITIZENSHIP **GB** |
| 2 | POST OFFICE ADDRESS | POST OFFICE ADDRESS **GlaxoSmithKline, Inc. Five Moore Drive, PO Box 13398** | CITY **Research Triangle Park** | STATE & ZIP CODE/COUNTRY **NC 27709 US** |

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